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Effect of metabolic status on conceptus-maternal interactions on Day 19 in dairy cattle: II. Effects on the endometrial transcriptome

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ABSTRACT

The aim of this study was to test the hypothesis that the metabolic stresses associated with lactation alter the ability of the endometrium to respond appropriately to the conceptus by examining endometrial gene expression on Day 19 of pregnancy. Immediately after calving, primiparous Holstein cows with similar production and fertility estimated breeding values (EBVs) were randomly divided into two groups and either dried off (*i.e.*, never milked) immediately or milked twice daily. Approximately 65-75 days postpartum, grade 1

blastocysts recovered from superovulated Holstein heifer donors (n=5) were transferred (1 per recipient) into lactating (n=11) and non-lactating (n=11) recipients. Control nulliparous Holstein heifers (n=6) were artificially inseminated. RNA-sequencing was performed on intercaruncular endometrial samples recovered at slaughter from confirmed pregnant animals on Day 19 (n=5 lactating and non-lactating cows; n=4 heifers). Differentially expressed genes (DEGs) were identified between both postpartum groups compared to heifers and between lactating and non-lactating cows. Functional annotation of DEGs between cows and heifers revealed over-representation of categories, including endosome, cytoplasmic vesicle, endocytosis, regulation of exocytosis and cytokine receptor activity. Functional categories including transcription factor binding sites, cell motility and cell migration were enriched for DEGs between endometria from lactating and non-lactating cows. In conclusion, while the evidence for a major effect of lactation on the endometrial transcriptome is relatively weak, these data suggest that the metabolic status of the animal (heifer vs cow) modulates the response of the endometrium to the developing conceptus.

INTRODUCTION

Intensive selection over recent decades for milk yield has come at a cost to reproductive performance in high-producing dairy cows. Although changes in the weightings in selection indexes in recent years have started to reverse this trend, the underlying causes of reduced fertility are still ambiguous. Whether the major impact of the metabolic perturbations associated with postpartum negative energy balance are at the level of the follicle, oocyte, embryo or reproductive tract environment is still unclear and is almost certainly multifactorial [1].

The reproductive tract of the postpartum lactating dairy cow is compromised in its ability to support early development compared to that of a nulliparous heifer [2] or a postpartum non-lactating cow [3]. This is associated with major differences in the profile of

circulating metabolites with lactation inducing high concentrations of nonesterified fatty acids (NEFA), beta hydroxybutyrate (BHBA), and low concentrations of insulin, IGF1 and glucose [3, 4].

The endometrial transcriptome can be altered by a range of factors including the presence of a conceptus [5, 6], circulating progesterone (P4) concentrations [7], lactation [8, 9], and various pathologies [10, 11]. Furthermore, reflecting the fact that preimplantation embryos are intrinsically diverse, there is an emerging concept of the endometrium as a biosensor, capable of responding differently to embryos of different quality or developmental fate [12-14]. This balance between endometrial receptivity and selectivity may reflect a maternal strategy to prevent inappropriate investment in embryos of poor viability [14].

There is substantial evidence in the literature that circulating P4 concentrations are significantly lower in post partum dairy cows compared to heifers [2, 15, 16]. Moreover, P4 concentrations in circulation modify the endometrial transcriptome as well as the ability of the uterus to support elongation [7, 17-19]. Conceptus length is correlated with interferon-tau (IFNT) production by the conceptus [20], which is a determining factor in the ability of the conceptus to successfully signal maternal recognition of pregnancy [21, 22]. The endometrium is also capable of distinguishing between conceptuses with different developmental trajectories, *i.e.* conceptuses produced by AI compared to those derived from *in vitro* produced or cloned embryos [12, 13], and modifies its transcriptomic response accordingly.

Previous studies have reported differences in the endometrial response to pregnancy in lactating cows compared to non-lactating cows [23, 24] following artificial insemination. Given the potential for a poor quality embryo to elicit a different response from the endometrium as described above, it is difficult to separate effects of lactation on the oocyte,

leading to the formation of a poor quality embryo, from those on the endometrium. Specifically, up to 50% of early pregnancy loss in dairy cows can be attributed to issues relating to the animals own oocyte/early embryo which can be overcome by the use of embryo transfer [25-27]. Thus, in order to remove potential confounding effects of the cow's own oocyte and to isolate the effects of the uterus, we used embryo transfer to test the hypothesis that the metabolic changes associated with lactation would impact the ability of the uterus to support conceptus elongation and appropriate pregnancy recognition signalling to the endometrium to establish an environment suitable for implantation. Specifically, we aimed to characterize the transcriptomic response of the endometrium to the developing conceptus during the peri-implantation period of pregnancy in postpartum lactating cows, postpartum non-lactating cows and nulliparous heifers.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. Unless otherwise stated, all chemicals and reagents were sourced from Sigma (Dublin, Ireland).

Animal model and sample collection

The metabolic profile of the animals used in this study has been previously described [28]. Briefly, 40 pregnant primiparous Holstein cows and 11 nulliparous Holstein heifers of similar estimated breeding value (EBV) were purchased. Immediately after calving, cows were either dried off (*i.e.* never milked, n=20) or milked twice daily, as is standard practice

(n=20). At approximately 65-75 days post partum (dpp) the estrous cycles of non-lactating (n=12), lactating cows (n=13) were synchronized by insertion of a controlled intra-vaginal drug releasing device (CIDR, Pfizer Animal Health, Sandwich, Kent, UK) containing 1.38 g of P4 for 8 days (Figure 1). One day prior to CIDR removal, all animals received a 2 ml intramuscular injection (i.m.) of a prostaglandin F2 alpha analogue (PG: Estrumate, Intervet, Dublin, Ireland; equivalent to 0.5 mg Cloprostenol) to regress the endogenous corpus luteum (CL). Thirty six hours after CIDR removal, each animal received a 2.5 ml i.m. injection of Receptal (Intervet, Dublin, Ireland: equivalent to 0.012 mg buserelin). In order to generate grade 1 embryos for transfer to the lactating and non-lactating recipient cows, nulliparous heifers (n=11) were synchronized as described above. Beginning on Day 10 following estrus detection (Day 0), each heifer received twice daily i.m. injections of Folltropin (Bioniche Animal Health, Ontario, Canada) given 12 h apart on a descending dose schedule (2.5 ml, 2.0 ml, 1.0 ml and 0.5 ml) for four days. Animals received a 2 ml i.m. injection of PG with the sixth injection of Folltropin. All superovulated donors were inseminated with semen from the same Holstein bull [VOLADI MAN FR4947788082] and were non-surgically flushed to recover embryos 7 days later. Prior to transfer all synchronised recipients were rectally palpated for the presence of a CL and, if present, one grade 1 late morula/early blastocyst was transferred into each lactating (n=11) and non-lactating (n=11) recipient. A control group of nulliparous heifers (n=6) was inseminated at standing estrus with semen from the same bull as above to generate tissues for comparison. On Day 19 following estrus, all animals (heifers and cows) were slaughtered at a commercial abattoir and the reproductive tracts were processed within 20 min. Each individual tract was stored on ice prior to sample collection. The uterine horn ipsilateral to the ovary bearing the CL was located and noted. Both uterine horns were flushed individually with 10 ml of phosphate buffered saline (PBS) and the recovered flush volume noted. The uterine luminal fluid (ULF) was then clarified by

centrifugation (1,000 rpm for 10 min at 4 °C), snap frozen in 1 ml aliquots in liquid nitrogen and stored at -80 °C prior to analysis. Endometrial tissue (caruncular and intercaruncular tissue were processed separately) from the mid-portion of the uterine horn ipsilateral to the ovary bearing the CL was dissected out from the underlying myometrium and also snap frozen in liquid nitrogen and stored at -80 °C prior to analysis.

Measurement of serum progesterone concentrations

In order to monitor the effect of treatment on serum P4 profiles, daily jugular blood samples were collected from Day 0 to 19 and stored at 4 °C for 24 h, spun at 1500 g at 4 °C for 20 min and the serum supernatant decanted and stored at -20 °C prior to analysis. Serum samples were analyzed for P4 concentrations as previously described [19] using the Coat-A-Count solid phase radioimmunoassay Progesterone kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) with an assay sensitivity of 0.03 ng/ml. The inter-assay coefficients of variation (% CV) were 10.1%, 11.9% and 1.7% for the low, medium and high quality control samples and the low, medium and high intra-assay CVs were 13.4%, 6.1% and 5.4%, respectively. The variables day following estrus, group, pregnancy status and their interactions were used to analyse differences in serum P4 concentrations using repeated-measures with the MIXED procedure of SAS (version 9.1.3; SAS Institute, Cary, NC, USA).

RNA-sequencing and data analysis

RNA was extracted from intercaruncular endometrial tissue from the pregnant uterine horn of animals confirmed pregnant at slaughter by the presence of a conceptus. RNA quality and quantity was confirmed using the Agilent Bioanalyser system and all RNA samples used for RNA sequencing had an RNA Integrity Number (RIN) of 7.9 or greater. Starting from

total RNA, stranded RNA sequencing libraries were constructed using the Encore Complete RNA-Seq library system of NuGEN. This protocol requires a minimum of 100 ng of total RNA and enriches for non-rRNA during cDNA synthesis. All libraries were sequenced on an Illumina HiSeq 1500 generating between 28 and 67 million 100 bp single-end reads per library.

The obtained sequence reads (Fastq files) were analyzed with several tools on a locally installed version of Galaxy [29]. First, the sequence reads were trimmed with Trimmomatic (v 0.33) (headcrop: 3 nt, trimming 3' end with sliding window size of 5 nt and quality cutoff = 30, and minlen: 30 nt). Several quality parameters of the fastq files were checked before and after trimming with FastQC (v0.11.2). Reads were mapped with Tophat2 (v2.0.11) [30] to the bovine genome sequence assembly (Btau_4.6.1, October 2011) and with the corresponding GFF annotation file from the National Center for Biotechnology Information (NCBI) (GCF_000003205.5). To count all mapped reads per gene we used the BioConductor package QuasR (v1.8.2) [31] within a modified R script. The read count table was then filtered in Microsoft Excel to remove genes with less than 20 reads in at least all 4 samples of the heifer group or 5 out of 6 samples of the non-lactating group or 4 out of 5 samples of the lactating group. Analysis of differential gene expression was performed with the BioConductor package EdgeR using the 'estimateGLMRobustDisp' [32]. A false discovery rate (FDR) of 5% was used as threshold for significance of differential gene expression for the comparisons heifer *vs.* non-lactating and heifer *vs.* lactating cows, and a FDR of 10% for lactating *vs.* non-lactating cows.

Venn diagrams were generated for genes with $p<0.001$ from all three comparisons using the web tool Venny 2.1 [33]. Hierarchical clustering was performed using MeV_4_8 v10.2 [34] for the differentially expressed genes (DEGs) based on log2 transformed and mean-centred counts per million (cpm) derived from EdgeR. DAVID Functional Annotation

Clustering [35] was used for the identification of overrepresented functional categories for the DEGs.

Measurement of glucose, lactate and pyruvate in uterine luminal fluid

Glucose, pyruvate, and lactate concentrations in ULF were measured as previously reported [36]. In brief, these carbohydrate substrates were quantified individually and indirectly via an enzymatic conversion producing a spectrophotometrically detectable nicotinamide byproduct. To achieve this, a FLUOstar Omega micro-plate reader (BMG LabTech; Ortenberg, Germany) was used, and samples were diluted to fit within standard curves. Initial substrate concentrations were determined by comparing fluorescence intensity against a standard curve and accounting for dilution. Statistically significant differences ($p<0.05$) were determined using Prism GraphPad 6 for Apple Macintosh, specifically by two-way analysis of variance (ANOVA) coupled with the Holm-Sidak non-parametric *post hoc* analysis.

RESULTS

Circulating concentrations of P4 increased significantly over time in all three groups however, there was no difference in serum P4 concentrations between the three groups i.e. heifers, non-lactating cows and lactating cows ($P>0.05$; Figure 2).

Differences in gene expression pattern

RNA-sequencing revealed between 28.4 and 67.1 million raw reads per sample. After filtering based on quality scores, between 28.2 and 65.7 million reads remained per sample

which were used for mapping to the bovine genome. The typical mapping rate of the reads to the genome was between 88 and 90%. The filtering based on a minimal read count for each gene resulted in 14,234 genes which were used for analysis in EdgeR [37].

Multidimensional scaling plots of endometrial data did not reveal a clear separation of the transcriptomic profiles of endometria from heifers compared to both postpartum groups, However, for principal component 1 the overall transcriptional profile of three of the heifer samples clustered together with one being more distant (Figure 3). No clear separation was observed for the samples from lactating and non-lactating cows in either the first nor in the second principal component.

Statistical analysis in EdgeR revealed 256 DEGs between heifers and non-lactating cows (FDR 5%, 131 increased and 125 decreased in heifers compared to non-lactating cows; Supplementary Table 1). The expression of transmembrane protein 130 (*TMEM130*) (6.3 fold), apomucin-like (*LOC104972555*) (5.3 fold), myelin protein P0-like (*LOC790208*) (4.8 fold), uncharacterized LOC100295797 (4.7 fold), and myelin protein zero (*MPZ*) (4.6 fold) were increased while the expression of uncharacterized LOC100847832 (8.3 fold), fucosyltransferase 6 (alpha (1,3) fucosyltransferase) (*FUT6*) (4.9 fold), uncharacterized LOC781494 (4.7 fold), Ig heavy chain V region PJ14-like (*LOC104968484*) (4.5 fold), and gamma-aminobutyric acid (GABA) A receptor, pi (*GABRP*) (4.4 fold) were decreased in the endometria of heifers compared to non-lactating cows to the greatest extent.

Comparing the endometrial transcriptome of heifers and lactating cows revealed 238 DEGs (FDR 5%), 141 of which were more abundant and 97 which were less abundant in heifers compared to lactating cows (Supplementary Table 2). Of these 238 DEGs, apomucin-like (*LOC104972555*) (9.9 fold), insulin-like growth factor 2 mRNA binding protein 3 (*IGF2BP3*) (5.8 fold), putative ankyrin repeat domain-containing protein ENSP00000383069

(*LOC513969*) (4.6 fold), uncharacterized LOC100295797 (4.5 fold), and vegetative cell wall protein gp1-like (*LOC104972646*) (3.9 fold) were increased to the greatest extent, while fucosyltransferase 6 (alpha (1,3) fucosyltransferase) (*FUT6*) (5.8 fold), sarcoglycan, delta (35kDa dystrophin-associated glycoprotein) (*SGCD*) (4.95 fold), glycosylphosphatidylinositol specific phospholipase D1 (*GPLD1*) (4.2 fold), multimerin 1 (*MMRN1*) (3.8 fold), and uncharacterized LOC781494 (3.7 fold) decreased to the greatest extent in endometria of heifers.

At a FDR of 10%, 28 DEGs were identified, 8 of which increased and 20 decreased, in endometria from lactating compared to non-lactating cows (Figure 4: Supplementary Table 3). A less stringent FDR was used for this comparison because of the lower number of genes showing expression differences which leads to a too strong correction of nominal P-values and a very low power to detect true DEGs [38]. The genes tenascin C (*TNC*) (3.3 fold), cancer antigen 1 (*CAGE1*) (2.7 fold), and anoctamin 3 (*ANO3*) (2.3 fold) were increased to the greatest extent, while solute carrier family 16, member 12 (*SLC16A12*) (4.9 fold), chemokine (C-C motif) ligand 5 (*CCL5*) (4.1 fold), and nuclear receptor subfamily 2, group E, member 1 (*NR2E1*) (3.2 fold) decreased to the greatest extent in endometria of lactating cows.

The overlap of the DEGs from the three comparisons (*i.e.* heifer *vs.* lactating, heifer *vs.* non-lactating and lactating *vs.* non-lactating) is shown in the Venn diagram in Figure 5. Since a different degree of correction for multiple testing (FDR) was observed for the three group comparisons, the nominal P-value was used as threshold for the genes used for the Venn diagram. An overlap of 92 genes was found for the comparisons heifer *vs.* lactating and heifer *vs.* non-lactating cows (Figure 5). Of these genes, 50 were increased in the endometria of heifers compared to both lactating and non-lactating cows while 42 were decreased in heifer endometria compared to both postpartum groups (Supplementary Table 4). Little overlap was

found for the DEGs between endometria from lactating and non-lactating cows with the comparisons to the heifer group.

DAVID Functional Annotation Clustering [35] was performed for the DEGs of each comparison. For heifer *vs.* non-lactating cow (Table 1) and heifer *vs.* lactating cow (Table 2) the analysis was done separately for genes with increased or decreased expression. Due to the low number of DEGs obtained for the comparison of endometria from lactating and non-lactating cows, up- and downregulated genes were analysed together (Table 3).

For the genes with lower expression in heifers compared to non-lactating cows functional categories such as 'vesicle', 'signal transduction', 'steroid metabolic process', and 'carbohydrate binding' were found as overrepresented (Table 1). Categories 'regulation of cell migration', 'cell death', and 'oxidoreductase' were enriched for genes with higher expression in heifers (Table 1).

Corresponding to the overlap of the DEGs for heifers *vs.* lactating cows and heifers *vs.* non-lactating cows, respectively, similar functional categories were found as overrepresented. Some additional categories were obtained, *e.g.*, genes with potential transcription factor binding sites for *HFH3*, *FREAC7*, and *RSRFC4* and 'cell adhesion' for genes with lower expression in heifers and 'fatty acid metabolic process', 'defence response', and 'lymphocyte activation' for genes with higher expression in heifers (Table 2).

For the DEGs lactating *vs.* non-lactating, only three overrepresented functional annotation clusters were obtained, which were related to response to hormone stimulus, cell migration, and genes with potential transcription factor binding sites for *IRF7*, *MYOD*, and *OCT* (Table 3).

Genes showing a P-value of <0.005 in comparison to lactating and non-lactating cows (137 genes) were compared to a number of other available data sets, *e.g.*, genes differentially expressed in bovine endometrium during the estrous cycle, on days 17 and 18 of pregnancy [5, 39], and in response to estrogen [40-42] (GEO GDS1510) (Figure 6). The latter geneset was used since it could also contain steroid hormone-regulated genes deregulated by metabolic stress. Forty seven genes out of these 137 genes were found to be differentially expressed in at least one of those three data sets. Most of the genes with higher expression levels at diestrus showed lower expression in endometria of lactating cows. The majority of overlapping DEGs found on days 17 and 18 of pregnancy were genes with lower levels in pregnant endometrium; about half of them had increased mRNA concentrations in endometria of lactating cows. Eleven of the overlapping genes have been found to respond to estrogen. For most of those genes expression was lower in endometria of lactating cows and also in response to estrogen or higher in lactating cows and after estrogen treatment.

Concentrations of glucose, lactate, and pyruvate in the ULF

Given the increased *SLC5A1* mRNA abundance in the endometrium of both dry and lactating cows compared to heifers, we hypothesised that difference in circulating glucose, along with *SLC5A1* expression in the endometrium, would contribute to differences in glucose, lactate and pyruvate availability in the uterine lumen. Concentrations of glucose in the ULF were not significantly different between heifers or dry and lactating cows (P>0.05: Figure 7) despite significantly higher *SLC5A1* expression in the intercaruncular endometrium of both dry and lactating cows compared to heifers (P<0.05). Lactate was significantly lower in the ULF of heifers compared to both dry and lactating cows (P<0.01 and P<0.001,

respectively). No significant difference in pyruvate concentrations in the ULF between the three groups was determined on Day 19 ($P>0.05$).

DISCUSSION

Lactation and the associated changes in energy balance during the post partum period has been shown to impact fertility and gene expression in the endometrium of postpartum high-yielding dairy cows [2, 9, 10]. In the study of Cerri *et al.* [9], endometrial gene expression was analysed in samples collected from Day 17 pregnant as well as cyclic lactating and non-lactating cows, respectively, and revealed a number of genes affected by lactational status indicating an effect of lactation on embryo-maternal crosstalk. The origin of this effect could be due to the effects of lactation and associated metabolic stress on the oocyte, the embryo, the reproductive tract or a combination of all three [43]. In order to remove confounding effects of lactation on oocyte quality and to isolate uterine effects, in this study, high quality embryos from superovulated donor dairy heifers were transferred to non-lactating (never milked after calving) and lactating post partum Holstein cows, respectively. As a control, a group of nulliparous Holstein heifers were inseminated to generate embryos and endometrium at the same stage.

We chose to analyse the endometrium on Day 19 for two main reasons. Firstly, it is the day when implantation is initiated in cattle and, as such, represents an important milestone for conceptus development. Secondly, and more importantly, two previous key studies have shown that the signal elicited by the conceptus from the endometrium around this time is strongly related with the subsequent development fate of the conceptus (Day 20: [12]; Day 18: [13]). Mansouri-Attia *et al.,* (2009) compared endometrial genes profiles on Day 20 in the presence of an in vivo fertilized embryo (AI) with those obtained in the presence of nuclear transfer (SCNT) or in vitro fertilized embryos

(IVF), both displaying lower and different potentials for term development. Their data provide evidence that the endometrium can be considered as a biological sensor able to fine-tune its physiology in response to the presence of embryos whose development will become altered much later after the implantation process. Bauersachs *et al.,* (2009) published a similar study in which we evaluated the response of the endometrium to SCNT embryos (produced from 7 different fetal fibroblast cell lines) compared with embryos derived from in vitro fertilization (IVF). SCNT embryos and IVF embryos were cultured under identical conditions to the blastocyst stage (Day 7) and, following transfer to recipients, were recovered at slaughter on Day 18 of pregnancy. The variation in mRNA profiles was greater in the SCNT group than in the IVF group and numerous transcripts were differentially abundant in endometria from SCNT and IVF pregnancies. The combined findings of these two studies suggest that placental failure in bovine cloned pregnancies may originate from abnormal embryo-maternal communication that develops during the peri-implantation period (Day 18-20). Furthermore, the data indicate that endometrium transcriptome profiles may serve as a tool to evaluate embryos for their ability to establish pregnancy and develop a functional placenta. Thus, the presence of a conceptus in the uterus on Day 18-20 is not a guarantee that the pregnancy will persist. Similar data regarding the 'sensory ability' of the endometrium have since been published for humans [14].

The results of this study have identified differences in endometrial gene expression between both lactating and non-lactating cows and heifers at the beginning of implantation. Hierarchical cluster analysis of the DEGs found in group comparisons indicates that endometrial gene expression is relatively similar in lactating and non-lactating cows. This finding is confirmed by the multidimensional scaling plot (principal component analysis) where samples from heifers were moderately more similar and separated from both lactating an non-lactating cow samples which were not discriminated by the PCA plot. The functional annotation of the DEGs between both postpartum cow groups and heifers revealed a number of overrepresented functional categories. Amongst the genes with lower expression in heifers,

genes related to “vesicle”, “cell adhesion”, and “steroid metabolic process” were found. The enrichment of genes assigned to the category “vesicle” may reflect differences in extracellular vesicle (EV) content or generation. EVs have been suggested to play an important role in embryo-maternal communication in sheep [44]. Some of the cell adhesion-related genes have been shown to be involved in trophoblast adhesion. The integrin heterodimer ITGB3/ITGB5 is important for the adhesion of human trophoblast cells to endometrial cells [45]. Hepatocyte growth factor (HGF) and its receptor MET have been implicated in placentation in the mouse [46] and CXCL12 in sheep [47]. Of the genes assigned to “steroid metabolic process” functional loss of *ABCA1* causes severe placental malformation in mice [48]. The glucocorticoid receptor *NR3C1* gene has been shown to be involved in regulation of endometrial functions during early pregnancy in sheep [49] and cattle [50].

The genes with higher expression in endometria of heifers were enriched for functional categories such as “defence response”, “lymphocyte activation”, “cell migration”, and “icosanoid biosynthetic process/fatty acid metabolic process”. The genes related to “defence response” have particularly interesting functions. For example, unc-13 homolog D (*UNC13D*) has been shown to play a role in vesicle maturation during exocytosis, in regulation of cytolytic granules secretion, and vesicle docking at the plasma membrane [51]. In the context of endometrial expression this could indicate a role in innate immune response and in vesicle trafficking in general. In addition to its inflammatory functions, a role for C-X-C motif chemokine ligand 3 (*CXCL3*) in trophoblast invasion has been suggested and aberrant expression of *CXCL3* might be involved in severe preeclampsia pathogenesis [52]. Another member of this family, *CXCL12*, involved in placenta formation [53] exhibited lower expression in the endometrium of heifers. Also, the expression of certain genes involved in the adaptive immune system was found to be higher in heifer *vs.* cow

endometrium, such as B-cell CLL/lymphoma 3 (*BCL3*) [54] and adenosine deaminase (*ADA*) [55]. *ADA* has been shown to play an essential role in early postimplantation development in mice [56]. Pentraxin 3 (*PTX3*) expression was also found to be higher in heifers and has been shown to be transiently expressed at implantation sites in mice indicating a role in implantation and decidualization. Furthermore, deletion of *Ptx3* lead to compromised implantation and decidualization [57]. Deleted in malignant brain tumors 1 (*DMBT1*) has been shown to be an estrogen-responsive gene and to be implicated in endometrial proliferation or differentiation in rodent and primate endometrial epithelium [58]. Advanced glycosylation end-product specific receptor (*AGER*) encodes a receptor for advanced glycation end products (AGEs) which are generated by modification of proteins, lipids, and nucleic acids by glucose. AGEs and other ligands interact with their receptor, AGER, which is mainly expressed in endothelium and vascular wall cells [59]. Elevated levels of serum AGER have been shown to be associated with recurrent pregnancy losses in women [60]. Arachidonate 5-lipoxygenase (ALOX5) is a key enzyme in the biosynthesis of leukotrienes, lipid mediators of inflammation generated from arachidonic acid [61]. Leukotrienes function in normal host defence and have pathophysiological roles in chronic inflammatory diseases [61]. Increased expression of *ALOX5* mRNA and protein was found in porcine uteri with endometritis induced by infection with *E. coli* [62]. CD163 belongs to the scavenger receptor cysteine-rich (SRCR) superfamily, and is exclusively expressed in monocytes and macrophages [63]. CD163 is induced by anti-inflammatory signals and downregulated in response to pro-inflammatory signals [63]. Upregulation of CD163 is associated with M2-activated macrophages which play a role in immune regulation, tissue remodelling, angiogenesis and apoptosis. In the cow, it has been shown that at least a part of endometrial macrophages differentiates along the M2 activation pathway during pregnancy [64].

In addition to “defence response” and other immune response-related functional categories, “regulation of cell migration” was overrepresented for genes with higher expression in heifer endometrium. Many of those genes are also involved in immune functions. Netrin 1 (*NTN1*) has been shown to be involved in the coordination of inflammatory responses by attenuation of neutrophil transepithelial migration [65]. Mucin 2 (*MUC2*) is known to be the main intestinal mucin involved in protection of the intestine and nutrition of commensal bacteria [66]. In human endometrium MUC2 expression has been detected during the secretory phase in glandular epithelium [67]. Similar functions have been shown for mucin 5AC, oligomeric mucus/gel-forming (*MUC5AC*), *i.e.*, protecting epithelial surfaces, growth, epithelial renewal and differentiation, and epithelial integrity [67, 68]. Knockout of *Mia3* in the mouse revealed an important function in secretion of collagen molecules and regulation of extracellular matrix composition [69]. For tropomyosin 1 (*TPM1*) temporal expression in the luminal epithelium has been shown in the peri-implantation mouse uterus [70].

The comparison of endometria derived from lactating and non-lactating cows, respectively, revealed only minor differences compared to those identified between heifers and the two postpartum cow groups. Although the model used by Cerri *et al.* [9] is in part comparable to the metabolic model of the present study, the comparison of the lists of DEGs revealed almost no overlap. The comparison to sets of DEGs identified in bovine endometrium during the estrous cycle [19], Days 17 and 18 of pregnancy [5, 39], and oestrogen-responsive genes [40] revealed some interesting overlaps. A number of genes with increased expression in endometrial samples of lactating cows were found as downregulated in pregnant endometrium in comparison to cyclic endometrium on Day 17 and/or 18. Among those genes were netrin 1 (see above), tenascin C (*TNC*), and SRY-box 5 (*SOX5*). Tenascin C

was found as DEG in bovine endometrium during the oestrous cycle with higher expression levels during oestrus [71] and also as upregulated by estradiol [40].

No significant differences were observed for glucose in the ULF. This was interesting given that both the dry and lactating cows expressed significantly higher mRNA for *SLC5A1* expression and given the fact that glucose in circulation was also higher than in heifers than in the dry and lactating group [4]. We propose that *SLC5A1* may act in a 'buffering' capacity ensuring that, irrespective of the concentrations of glucose in circulation, similar concentrations are available in the ULF to the pre-implantation conceptus. Further evidence supporting this hypothesis is provided by the study of [72] whereby infusion of glucose did not alter uterine glucose composition.

In conclusion, transfer of high quality embryos into the uterus of lactating and non-lactating dairy cows did not have a major impact on the endometrial transcriptomic response; the largest differences observed in mRNA levels in the endometrium occurred when these two groups were compared to maiden heifers with a minimal effect on those genes that are involved in the classical pregnancy recognition signal in the endometrium. It may be that lactation does not affect the type 1 interferon response in the endometrium to the conceptus but may affect other signals that are involved in pregnancy recognition and or establishing uterine receptivity to implantation [73-75]. These data do not support the hypothesis that that the metabolic changes associated with lactation substantially altered the ability of the uterus to support conceptus elongation and appropriate pregnancy recognition signalling but identified more substantial alterations to the endometrium of the heifer compared to both lactating and non-lactating cows.

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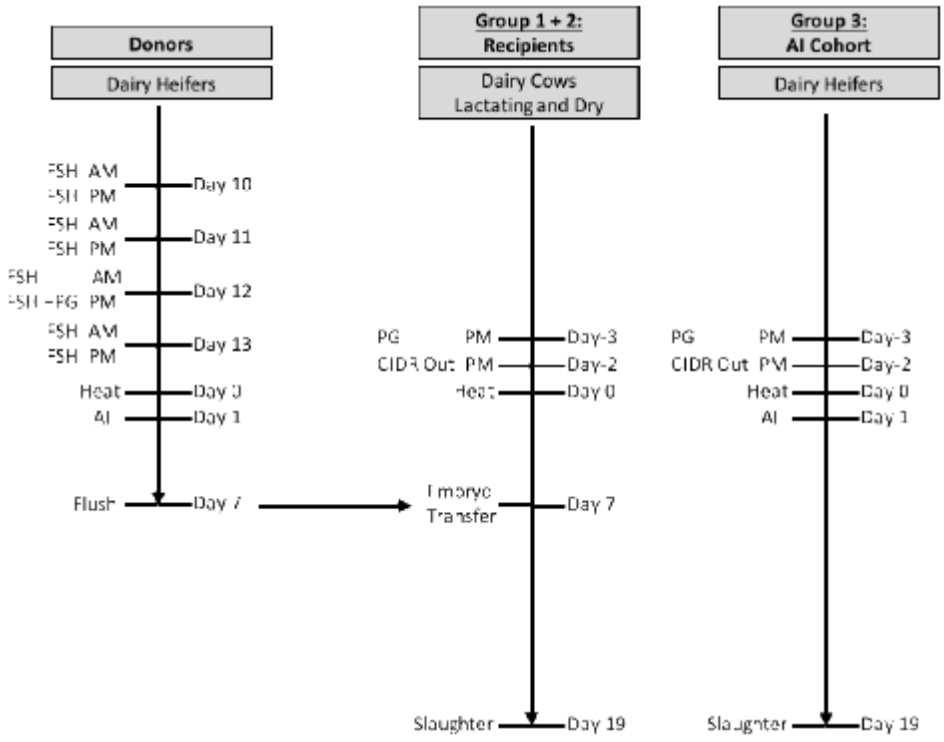

Figure 1. Schematic diagram of the animal model used to generate intercaruncular endometria from heifers (n=4), lactating (n=5) and non-lactating (n=5) dairy cows on Day 19 of pregnancy. FSH = Follicle stimulating hormone; PG = prostaglandin analogue; CIDR = Controlled Intravaginal Drug Releasing device; AI = Artificial Insemination.

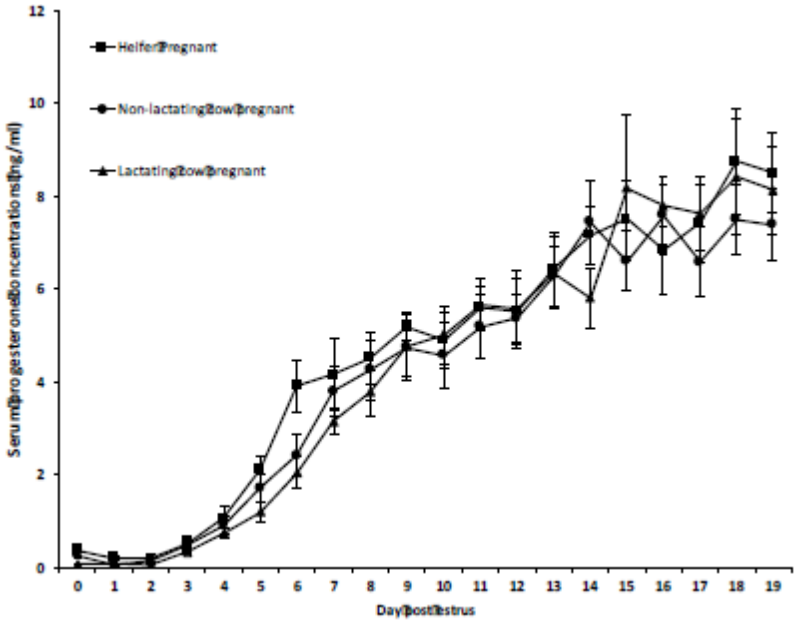

Figure 2. Circulating concentrations of P4 (ng/ml) in heifers confirmed pregnant (black square), non-lactating cows confirmed pregnant (black circle), and lactating cows confirmed pregnant (black triangle) on Day 19 following estrus. Serum P4 concentrations increased significantly in all three groups as pregnancy progressed ($P<0.05$) however, there was no significant difference in P4 concentrations between lactating cows, non-lactating cows and/or maiden heifers ($P>0.05$).

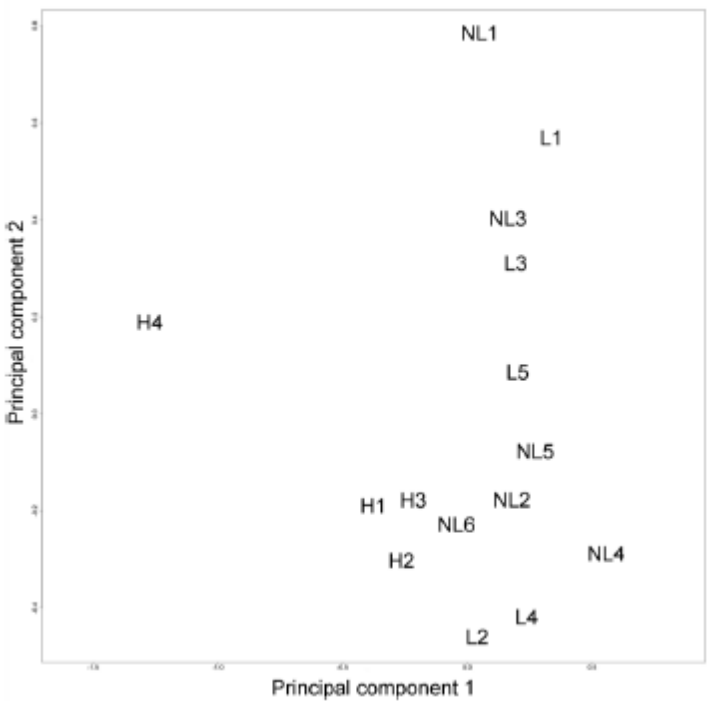

Figure 3. Multidimensional scaling plot. A multidimensional scaling plot (principal component analysis) for the top 500 genes showing the highest pairwise fold changes between the samples in the dataset was performed in EdgeR. L: lactating cows, NL: non-lactating cows, H: heifers.

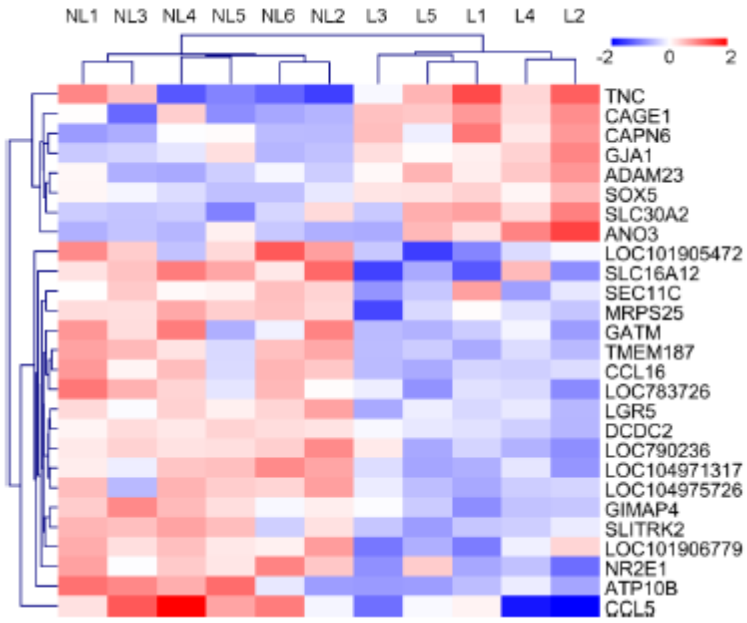

Figure 4. Hierarchical cluster analysis of DEGs found for lactating *vs.* non-lactating cows. Mean-centered expression values (log2 scale) were calculated for genes with P <0.005 und used for hierarchical cluster analysis (MeV_4_8 v10.2). NL: non-lactating; L: lactating.

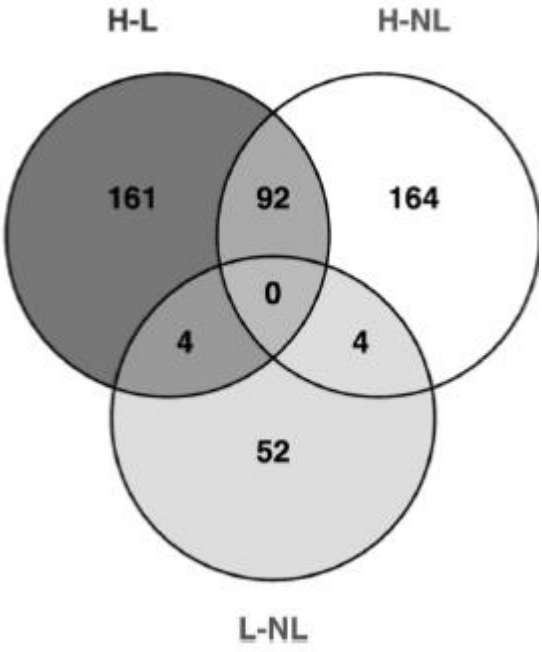

Figure 5. Venn diagram for the overlap of DEGs between all three group comparisons, i.e. the differentially expressed genes that were common or distinct to each of the three comparisons made. L: lactating cows, NL: non-lactating cows, H: heifers. Venn diagram analysis was performed by jvenn [76] for the DEGs in each comparison that had a nominal P-value <0.001.

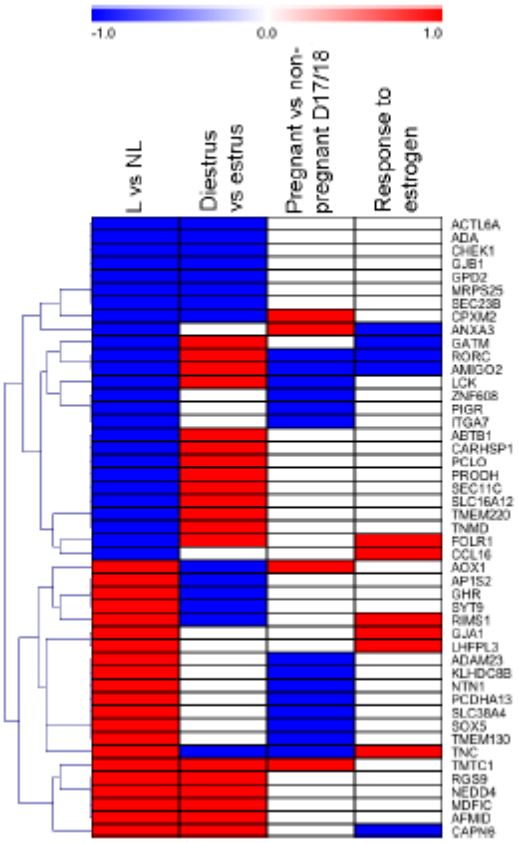

Figure 6. Comparison of the differentially expressed genes in endometria of lactating *vs.* non-lactating cows with results from other studies. Genes with a P-value of <0.005 were compared to lists of genes found as differentially expressed in bovine endometrium during the estrous cycle and early pregnancy as well as genes known or inferred to be regulated by estrogen. Red: increased expression, Blue: decreased expression (log2 fold), White: no match, L: lactating cows, NL, non-lactating cows , D: day.

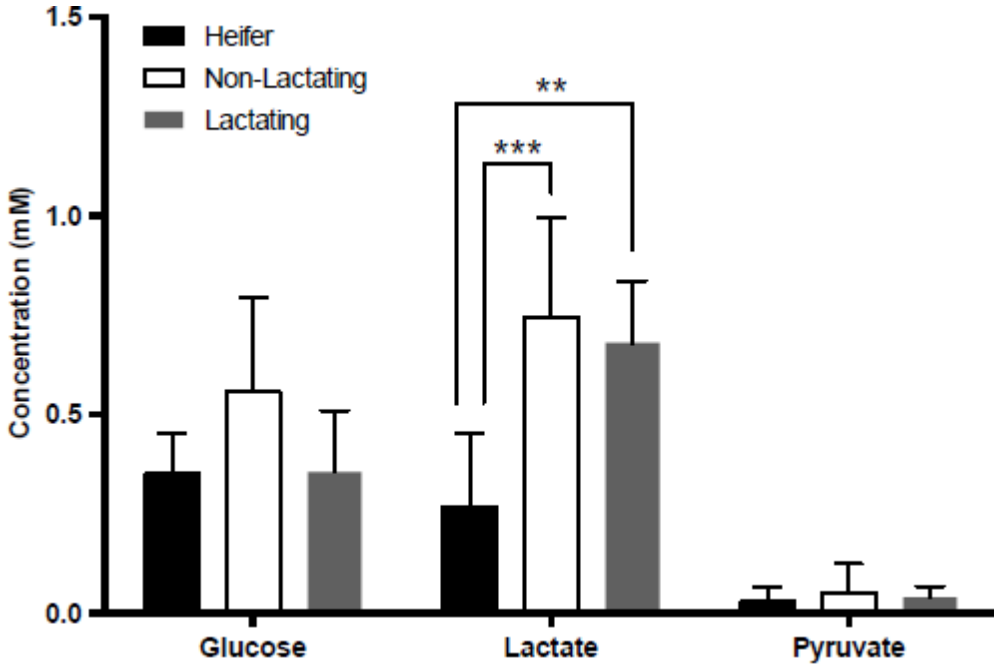

Figure 7. Mean glucose, lactate, and pyruvate concentrations in the uterine luminal fluid of lactating cows (black bar; n=5 ± SD), dry cows (open bar; n=6 ± SD), and maiden heifers (grey bar; n=4 ± SD) confirmed pregnant on Day 19.

Table 1: DAVID functional annotation clusters with a score ≥1.3 for DEGs heifer vs. non-lactating cow

Most descriptive categories of DAVID functional annotation clusters	# Genes	Score[1]
Genes with lower expression in heifers vs. non-lactating cows		
membrane (59, 1.6)[2], integral to membrane (53, 1.4)	60	3.56
vesicle (15, 3.0), cytoplasmic membrane-bounded vesicle (11, 2.7)	15	2.72
cell surface receptor linked signal transduction (17, 1.3), transmembrane receptor protein tyrosine kinase signaling pathway (9, 5.9)	17	2.65
glycoprotein (46, 1.8), signal peptide (30, 1.6), disulfide bond (24, 1.4)	50	2.57
plasma membrane (37, 1.3), integral to plasma membrane (17, 1.9)	40	1.86
transmembrane receptor protein tyrosine kinase signaling pathway (9, 5.9), protein tyrosine kinase activity (5, 4.7), axon guidance (5, 4.5)	14	1.77
anion transmembrane transporter activity (4, 4.3), ABC transporters (3, 7.9)	4	1.54
steroid metabolic process (6, 4.4), cholesterol metabolic process (4, 6.4), lipid localization (4, 3.7)	7	1.53
carbohydrate binding (6, 2.7), glycosaminoglycan binding (4, 4.5)	6	1.50
homeostatic process (9, 1.8), response to hypoxia (4, 4.4)	10	1.36
Genes with higher expression in heifers vs. non-lactating cows		
membrane fraction (13, 3.1)	14	2.85
regulation of locomotion (6, 5.6), regulation of cell migration (5, 5.3), negative regulation of cell motion (4, 11.5)	6	2.17

plasma membrane part (19, 1.6), integral to plasma membrane (12, 1.9)	19	1.55
cell death (10, 2.5), apoptosis (8, 2.4)	10	1.42
oxidoreductase (6, 2.1), icosanoid metabolic process (3, 11.5)	6	1.30

[1]geometric mean of member's p-values of the corresponding annotation cluster (in -$\log_{10}$ scale); [2]in brackets: number of genes and fold enrichment for the functional term.

Table 2: DAVID functional annotation clusters with a score ≥1.3 for DEGs heifer vs. lactating cow

Most descriptive categories of DAVID functional annotation clusters	# Genes	Score[1]
Genes with lower expression in heifers vs. lactating cows		
transcription factor binding sites for HFH3 (40, 1.5)[2], FREAC7 (44, 1.4), RSRFC4 (42, 1.4)	53	2.51
membrane (38, 1.5), integral to membrane (34, 1.4)	38	2.35
cytoplasmic vesicle (10, 3.5), cytoplasmic membrane-bounded vesicle (8, 3.3)	10	2.33
glycoprotein (30, 1.7), signal peptide (21, 1.6), disulfide bond (18, 1.5)	32	1.74
cell adhesion (9, 2.9)	9	1.69
transmembrane protein (6, 2.3), Focal adhesion (5, 4.7)	8	1.55
protein kinase cascade (5, 3.1), regulation of protein kinase activity (5, 3.3), activation of MAPK activity (4, 11.2)	6	1.36
EGF-like region, conserved site (6, 4.7), EGF (4, 4.0)	6	1.36
plasma membrane (22, 1.3), extracellular (18, 1.6)	29	1.31
Genes with higher expression in heifers vs. lactating cows		
glycoprotein (34, 1.4), signal peptide (33, 1.8), extracellular region (22, 1.6)	40	2.33
von Willebrand factor, type C (3, 12.9), cystine knot, C-terminal (3, 20.6)	3	1.95
fatty acid metabolic process (5, 4.1), icosanoid biosynthetic process (3, 15.8)	6	1.42
defense response (9, 2.4), inflammatory response (6, 3.0)	11	1.40
lymphocyte activation (4, 3.3), T cell activation (3, 3.9)	4	1.35
carbohydrate binding (7, 3.2), polysaccharide binding (4, 4.2)	7	1.34

[1]geometric mean of member's p-values of the corresponding annotation cluster (in -log_{10} scale); [2]in brackets: number of genes and fold enrichment for the functional term.

Table 3: DAVID functional annotation clusters with a score ≥1.3 for DEGs lactating vs. non-lactating cow

Most descriptive categories of DAVID functional annotation clusters	# Genes	Score[1]
response to peptide hormone stimulus (3, 18.8)[2], response to oxidative stress (3, 17.7)	3	1.48
cell motility (3, 9.4), cell migration (3, 10.5)	3	1.41
transcription factor binding sites for IRF7 (12, 2.0), MYOD (11, 1.5), OCT (9, 1.5)	13	1.32

[1]geometric mean of member's p-values of the corresponding annotation cluster (in -log_{10} scale); [2]in brackets: number of genes and fold enrichment for the functional term.